Chapter 4
The Use of Genetically Engineered Mice to Study PAD Biology and Pathology

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4.1 Introduction

Peptidylarginine deiminases (PADs or PADIs) are a family of calcium-dependent enzymes that post-translationally convert positively charged arginine residues to neutrally charged citrulline in a process called citrullination or deimination. There are five PAD family members (PAD1–PAD4 and PAD6). PAD genes arose by duplication and are clustered within a ~300-kb region on chromosome 1p36 in humans and within a ~230-kb region on chromosome 4 in mice. In both species PAD1, PAD3, PAD4, and PAD6 are grouped closely together and are oriented in the same direction, while PAD2 is set apart from the other PADs by at least 60 kb and is oriented in the opposite direction (Vossenaar et al. 2003). PAD isozymes are expressed in a range of tissues in mammals, with PAD2 being broadly expressed in numerous tissues, while PAD4 is highly represented in immune cells. PAD6 expression, on the other hand, is primarily limited to oocytes and early embryos, whereas PAD1 and PAD3 appear to be mainly expressed in the epidermis. While still coming to light, functional roles for PADs in mammalian physiology and pathology are diverse and include cellular differentiation, nerve growth, apoptosis, inflammation, gene regulation, and early embryonic development. Over the last several years, investigators have generated genetically engineered mice (GEM) for PAD2, PAD3, PAD4, and PAD6 to investigate the functions of these unique enzymes at the organismal level. Outcomes from these studies are highlighted in Fig. 4.1, and the goal of this chapter is to provide a broad summary of findings obtained from these animals.

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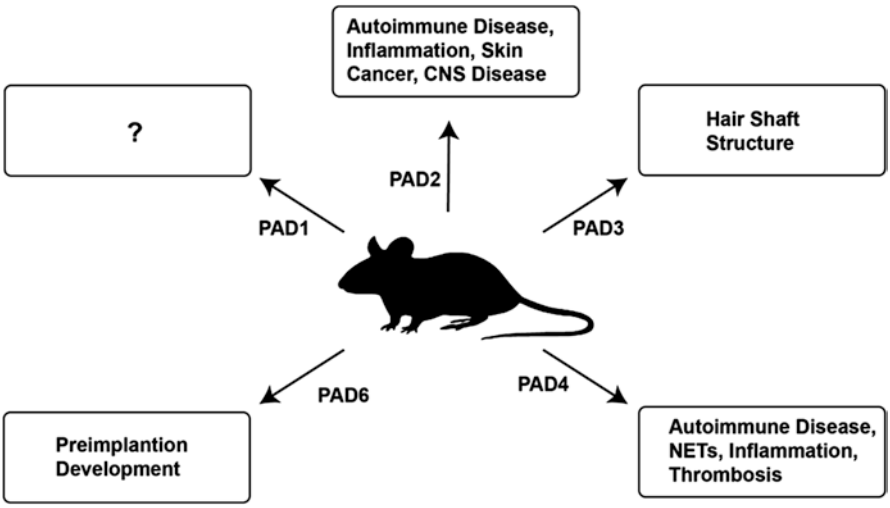

Fig. 4.1 Specific physiological and pathological functions that have been given to PAD isozymes based on outcomes from studies using genetically engineered mice

4.2 PAD2

PAD2 appears to be the most broadly expressed of the PADs, with its expression in humans being reported in muscles, nervous tissue, skin, immune cells, and secretory epithelia (including the mammary gland, uterus, and salivary glands) (Horibata et al. 2012). Most of the earlier reports on PAD2, however, focused on its role in citrullinating myelin basic protein (MBP) during the inflammatory phase of multiple sclerosis (MS) (Akiyama et al. 1999). To determine the extent to which PAD2-mediated protein citrullination contributes to this process, Raijmakers et al. tested the effects of PAD2 deletion on induced experimental autoimmune encephalomyelitis (EAE) in mice (Raijmakers et al. 2006). These mice were generated by disrupting a 164-bp region of exon 1 using a targeting vector which was derived from the Lambda KOS system (Raijmakers et al. 2006). The investigators first validated the knockout and demonstrated that PAD2 protein was lost in the brain and spinal cord of the null mice. While not shown, the investigators also noted that the expression levels of PAD1, PAD3, and PAD4 were not affected by PAD2 deletion. They then tested the effects of loss of PAD2 on EAE and found that, while PAD2 is required for citrullination in nervous tissue, PAD2 does not appear to be required for experimentally induced EAE. Importantly, PAD2 deletion also did not appear to affect brain or spinal cord development, suggesting that, while PAD2 is abundantly expressed in the nervous system, it is not required for normal function in these tissues (Raijmakers et al. 2006). These studies support the hypothesis that while PAD2 is the main PAD isozyme that is responsible for citrullination in the CNS in both healthy and EAE mice, this activity does not play a critical role in experimentally induced EAE. In a separate study using the same PAD2-null mouse line,

investigators demonstrated that deimination is seen in isolated myelin of PAD2 knockout mice and predict that this citrullination activity may actually be catalyzed by PAD4 (Wood et al. 2008).

In another study aimed at investigating the role of PAD2-mediated citrullination of MBP in MS, Musse et al. (2008) developed a mouse line that contained multiple copies of rat PAD2 cDNA under the control of the MBP promoter. Following validation of transgene overexpression in the CNS, the investigators documented clinical disease development over a 6-month period following birth. They found that clinical signs of demyelinating disease occurred at a younger age in PAD2-overexpressing mice when compared to controls. The investigators then tested whether the symptoms were caused by increased PAD2 activity in the brain. They found that PAD2 protein levels were elevated in the white matter of the PAD2-overexpressing mice and that citrullination of MBP was increased at these sites as well. Additionally, the investigators found that subtle changes in axon and myelin thickness were seen in the PAD2-overexpressing mice. Interestingly, PAD4 mRNA levels were found to be increased by fourfold in PAD2 heterozygous mice and a further fivefold in PAD2 homozygous mice. The investigators also reported that histone citrullination and nuclear PAD4 were significantly elevated in the PAD2-overexpressing mice, as were tumor necrosis factor alpha (TNFα) levels (Musse et al. 2008). Taken together, these findings suggest that PAD2 may play a role in myelin destabilization during MS progression and also raise the possibility that PAD2 cooperates with PAD4 to mediate this activity.

PAD2 and PAD4 are often found to be co-expressed in specific cell types, particularly immune cells, and both of these PADs are implicated in various pathologies including inflammation, autoimmune disease, and cancer. In order to begin to tease out specific functions for these two family members, a recent study made use of PAD2 and PAD4 mutant mice to investigate the expression and activity of these isozymes in various murine tissues (van Beers et al. 2013). In this particular study, the PAD4 mutant line was generated via insertional mutagenesis, which resulted in the insertion of a 156-bp cassette in intron 1 of the PAD4 gene. Subsequent PCR and Western blot analysis found that this mutant line was actually a partial knockout, and these mice retained a low level of PAD4 protein (van Beers et al. 2013). The PAD2-null mice used in this study were the same line as that used by Raijmakers et al. and were true knockouts. The investigators went on to document citrullination levels in these mutant mice in various tissues (including the brain, lung, spleen, etc.) using an antibody-based assay for PAD activity (ABAP) and an anti-modified citrulline antibody. Results show that PAD activity was virtually absent in a range of tissues from PAD2-null mice while PAD activity in the PAD4-low mice was similar to that of controls. The investigators then measured PAD activity in peripheral blood mononuclear cells and in polymorphonuclear leukocytes and found that loss of PAD2 only moderately affected citrullination levels in immune cells. These results suggest that the contribution of PAD2 to overall PAD activity in white blood cells is not as pronounced as that seen in other tissues. Interestingly, these investigators also found that PAD6 levels were increased PAD2-null and PAD4-low mouse tissue. This surprising result raises the possibility that PAD6 (which is normally expressed

in oocytes and early embryos) may compensate for the loss of other PADs in somatic tissues. This prediction does not seem likely, however, since PAD6 does not appear to be catalytically active (Taki et al. 2011).

Similar to PAD4, PAD2 has been documented to be expressed in T cells (Ferrari-Lacraz et al. 2012), neutrophils (Darrah et al. 2012), and macrophages (Vossenaar et al. 2004). While the role of PAD4 in immunological processes such as inflammation and autoimmune disease has received most of the attention, PAD2 has also been implicated in these processes (Foulquier et al. 2007; Damgaard et al. 2015). In order to more directly investigate and compare the roles of PAD2 and PAD4 in inflammation, a recent study investigated the effect of PAD2 or PAD4 deletion on inflammation using a TNF-overexpressing mouse model of inflammatory arthritis (Bawadekar et al. 2017). For the study, the investigators crossed TNF-overexpressing mice with either PAD2- or PAD4-null lines and then investigated the effects of PAD deletion on citrullination and ankle joint inflammation. The PAD2- and PAD4-null mouse lines used for this study were generated by other groups (Raijmakers et al. 2006; Li et al. 2010), and both appear to be complete knockouts. Surprisingly, results show that PAD2, but not PAD4, is required for gross protein citrullination in the inflamed arthritic joints of these mice. Further, they found that PAD2 is required for maximal severity of TNF-induced arthritis in their mouse model. PAD4 has previously been found to be required for neutrophil extracellular trap (NET) production via its role in histone citrullination (Li et al. 2010; Wang et al. 2009). Given the previously established links between NETs and inflammation, the investigators tested to see if PAD2 might also be required for NET production (note: a more detailed discussion on NETs is found in Sect. 4.4 below). Results show that, while the PAD4-null mice could not produce NETs, production of this inflammation-promoting structure was not affected by PAD2 deletion. Taken together, these studies provide the most direct evidence that PAD2 contributes to inflammatory arthritis. Additionally, these findings suggest that NETs may not be the main source of citrullinated proteins in arthritic mice and raise the possibility that PAD4's role in inflammation may be something other than direct citrullination of antigens. Lastly, given the requirement of PAD2 for generating citrullinated proteins in this model system, these results suggest that PAD2 may play an even larger role in autoimmune diseases than previously expected.

In addition to nervous tissue and immune cells, PAD2 is also expressed in the skin. Previous reports have found that PAD1, PAD2, and PAD3 are expressed in the epidermis, with PAD2 expression being primarily found within the spinous and granular layers of the mouse epidermis (Ying et al. 2009). Interestingly, a recent analysis of the skin in PAD2-null mice found that loss of PAD2 does not appear to affect citrullination levels in the skin of mice, nor did PAD2 deletion appear to affect PAD1 and PAD3 levels in the epidermis (Coudane et al. 2011).

Over the last several years, we have been investigating the role of PAD2 in breast cancer using cell lines and mouse xenografts. In order to further investigate these links, we recently generated a FVB/N mouse that overexpressed human FLAG-PAD2 under control of the mammary tumor virus (MMTV) promoter (McElwee et al. 2014). Our results demonstrated that, similar to previous studies, MMTV drove the expression of PAD2 in a range of tissues including the mammary gland, salivary

gland, ovaries, and skin. Interestingly, we found that, while this mouse line did not develop overt signs of mammary cancer, ~40% of the transgenic mice developed skin lesions between 4 and 12 months, with a subset of these lesions progressing to squamous cell carcinoma (SCC). At a more mechanistic level, we found that the expression of inflammatory markers such as IL6 and Cox2 was significantly increased in the skin of PAD2-overexpressing mice and also in PAD2-overexpressing SCC cell lines (McElwee et al. 2014). As noted earlier, previous studies have shown that, in some tissues, either deletion or overexpression of one PAD can alter the expression of other PADs. In our study, we found that the expression of PAD1–PAD4 appeared to be significantly dysregulated in several tissues in the PAD2-overexpressing mice. These results provided the first genetic evidence that PAD enzymes can function as oncogenes and also suggested that PAD2 may promote oncogenesis via its role as an inflammatory mediator. Given that PAD2 overexpression altered the expression of other PAD family members, we cannot rule out the possibility that some of the effects observed in our study were due to compensation by other PADs.

4.3 PAD3

As noted above, PAD3 appears to primarily be expressed in the epidermis and hair follicles. Recent studies have found that mutation of the PAD3 and TGM3 (transglutaminase 3) genes in humans causes uncomfortable hair syndrome (UHS), also known as "spun hair glass syndrome" (Ü Basmanav et al. 2016). This rare disease is an anomaly of the hair shaft and is characterized by dry frizzy hair that is difficult to comb flat. The prediction that PAD3 mutations cause UHS in humans was supported by the investigators finding that deletion of PAD3 resulted in subtle alterations in the hair shaft in these animals. Results showed that, while these mice were viable and had grossly normal skin, SEM analysis of their skin found that hair shafts from these mice were irregular, rough, and appeared as if "hammered" (Ü Basmanav et al. 2016). The investigators then generated a mutant construct for cell culture studies and found that the putative causative mutation significantly reduced PAD3 activity in vitro, suggesting that PAD3 activity was required for the observed defect. The investigators also noted that, while PAD3 is highly expressed in the hair follicle and upper epidermis, there was no observable phenotype in the epidermis of the null mice. While not tested, the investigators speculated that this might be due to compensation by other PADs.

4.4 PAD4

Given PAD4's close ties with rheumatoid arthritis (RA), which affects ~1% of the world's population (Begovich et al. 2004), this PAD family member has received most of the attention in the biomedical arena. Individuals with RA frequently have

autoantibodies to citrullinated peptides (van Gaalen et al. 2004) and several studies have identified PAD4 as a susceptibility locus for RA (Suzuki et al. 2003). Additionally, as noted above, PAD4 has also been found to be involved in neutrophil extracellular trap (NET) production (Wang et al. 2009). These chromatin-based structures are released by neutrophils as part of the innate immune system to bind to and kill pathogens (Brinkmann and Zychlinsky 2012). Importantly, NETs have also been found to be a source of citrullinated autoantigens, which can promote RA symptoms (Khandpur et al. 2013). Aside from RA, dysregulated NET activity has been associated with a range of disease states, including vasculitis, atherosclerosis, and thrombosis (Kaplan and Radic 2012). Recently, investigators have begun studying PAD4-null mouse lines to strengthen links between PAD4 and these disease states and also to investigate the mechanisms by which PAD4 modulates the immune system.

In one of the first reports using PAD4 knockout mice, Li et al. (2010) generated a PAD4-null line by deleting exon II from PAD4, thus causing premature termination of the PAD4 transcript. The investigators then confirmed that PAD4 protein was absent from neutrophils in these mice and found that, despite the loss of PAD4, the null mice were viable with no detectable gross physical abnormalities. The investigators then demonstrated that histone H3 citrullination was abolished in neutrophils from PAD4-null mice and that these neutrophils were unable to undergo NET production following stimulation with chemokines or bacteria. The investigators then utilized a mouse model of necrotizing fasciitis to demonstrate that PAD4-null mice are more susceptible to bacterial infection. The investigators concluded that this deficiency was likely due to a failure to produce NETs at the site of infection (Li et al. 2010). These important studies provided the first genetic evidence that PAD4 is an important immune mediator that is required for NET-mediated antibacterial innate immunity.

A separate study by Hemmers et al. generated another PAD4 knockout line by introducing loxP sites that flanked exons 9 and 10 (which encode the active site of PAD4), and the site was removed by mating the mice with CMV-Cre deleter mice. Similar to the study by Li et al., the investigators found that histone citrullination and NET production was not detectable in PAD4-null neutrophils. These investigators also found that PAD4 deficiency in neutrophils does not impact leukocyte recruitment to the lungs of influenza-challenged mice. These results suggest that, while PAD4-mediated NET production plays an important role in antibacterial immunity, PAD4-mediated NET formation appears to be dispensable during viral infection (Hemmers et al. 2011).

A report by Rohrbach et al. used the PAD4 knockout line generated by Hemmers et al. to test the requirement of PAD4 for autoimmune disease using a K/BxN autoantibody-mediated model of arthritis (Rohrbach et al. 2012). In this model, autoantibody-containing serum from K/BxN mice (which spontaneously develop a progressive inflammatory joint disease) was injected into the peritoneum of wild-type or PAD4-null mice, to stimulate autoantibody-mediated arthritis. Results show that, similar to previous studies, PAD4 activity and NET formation was absent in neutrophils from PAD4-null mice. However, both wild-type and PAD4-null mice developed K/BxN-induced inflammatory arthritis, suggesting that PAD4 is dispensable for the effector phase of this disease (Rohrbach et al. 2012).

TNFα is known to play a critical role in RA and, more generally, in inflammatory arthritis (Feldmann and Maini 2003). Additionally, TNFα can also induce the nuclear translocation of PAD4 , histone citrullination, and NET formation (Mastronardi et al. 2006). It has also been shown that citrullinated proteins and anti-citrullinated protein antibodies (ACPAs) can induce TNFα production by macrophages (Sokolove et al. 2011). Given these interconnections, investigators recently crossed TNFα-overexpressing mice with wild-type and PAD4-null mice in order to test the hypothesis that PAD4 may regulate TNFα-mediated autoantibody production and inflammatory arthritis (Shelef et al. 2014). Results showed that TNFα-overexpressing wild-type mice displayed increased levels of autoantibodies that were reactive against native and citrullinated antigens when compared to the TNFα-overexpressing PAD4-null mice. Additionally, the TNFα-overexpressing PAD4-null mice also displayed reduced inflammation and arthritis when compared to TNFα-overexpressing wild-type PAD4 mice. These results suggest that PAD4 mediates autoantibody production and inflammatory arthritis downstream of TNFα (Shelef et al. 2014). The important role that PAD4 plays in promoting inflammatory arthritis has also been supported by several recent studies showing that arthritis severity is significantly reduced in PAD4 knockout mice using glucose-6-phosphate (Seri et al. 2015) and collagen-induced (Suzuki et al. 2016) models of inflammation.

Given that PAD4-null mice do not produce NETs, these mice have also been utilized to investigate the role of NETs in placentation disorders (Erpenbeck et al. 2016). Results from these studies found that overexpression of soluble fms-like tyrosine kinase (sFlt-1, which has been associated with abnormal placentation disorders during early gestation) resulted in miscarriage and the accumulation of neutrophils and NETs in the placentas of wild-type PAD4 mice. However, neutrophil invasion, NET production, inflammation, and pregnancy losses were significantly abrogated in PAD4-null mice that overexpressed sFlt-1. The investigators then went on to show that neutrophil invasion and NET production was significantly higher in preeclamptic women compared to non-hypertensive controls (Erpenbeck et al. 2016). These findings suggest that PAD4-specific inhibitors may have therapeutic potential for the treatment of preecplampsia in women.

NETs have recently been shown to be involved in thrombosis formation via their role in generating the thrombus scaffold and promoting coagulation (Martinod and Wagner 2014). To investigate the mechanism by which PAD4-mediated citrullination mediates this process, Martinod et al. recently tested the effects of PAD4 deletion on thrombus formation using a mouse venous stenosis model of deep vein thrombosis. The study found that <10% of the PAD4-null mice produced a thrombus, compared to 90% of wild-type mice which generated a thrombus. Interestingly, the investigators found that thrombosis could be rescued by infusion of wild-type neutrophils, suggesting that neutrophil PAD4 was sufficient for the observed effect. These results strongly suggests that NETs represent a crucial component of the thrombus scaffold (Martinod et al. 2013).

Acute myocardial infarction (AMI) is a major component of cardiovascular disease and is caused by intraluminal coronary thrombosis. AMI can be modeled in

mice using an ischemia and reperfusion (I/R) technique. To test whether PAD4-mediated citrullination may also play a role in this type of thrombosis, Savchenko et al. examined the effect of I/R on thrombus production in PAD4-null mice (Savchenko et al. 2014). Results showed that the mice (which do not produce NETs) were significantly protected from I/R treatment. The study also found that PAD4 deficiency reduced leukocyte recruitment to the infarcted myocardium and prevented nuclear histone citrullination at this site (Savchenko et al. 2014).

Outcomes from these studies suggested that PAD4 inhibitors could have therapeutic benefit for patients with thrombosis and ischemic/reperfusion injury. A follow-up study by Martinod et al. (Martinod et al. 2015) investigated the role of NETs in sepsis, with respect to the balance between their antimicrobial and cytotoxic actions (Martinod et al. 2015). Given that PAD4 is required for NET production and that NETs may have important antimicrobial capabilities, this study was carried out because there was concern that the therapeutic benefit of PAD4 inhibitors may be offset by the possibility that these inhibitors might promote sepsis. The investigators addressed this concern by inducing sepsis in PAD4 KO mice using cecal ligation and puncture. Results showed that survival was comparable between PAD4 KO and wild-type mice. They also found that neutrophil functions involved in bacterial killing (other than NETosis) remained intact in the PAD4 KO mice. Outcomes from these studies suggested that preventing NET formation by PAD4 inhibition in inflammatory or thrombotic diseases will not likely increase the patients risk for bacterial infections (Martinod et al. 2015).

In another study aimed at investigating antimicrobial effects of PAD4 and NETs, Kolaczkowska et al. found that, during bloodstream infection with *S. aureus*, most bacteria are sequestered within liver sinusoids by Kupffer cells and that this sequestration promotes ischemia and neutrophil infiltration into the area (Kolaczkowska et al. 2015). The investigators then found that the sequestered neutrophils release NETs (which contain high levels of neutrophil elastase) into the sinusoids and these NETs then become anchored to the endothelium by binding to von Willebrand factor (VWF). Next, the investigators showed that, while DNase is highly efficient at removing DNA from the NETs, elastase and histones (which are highly cytotoxic) remain associated with the NETs and likely promoted severe tissue damage. Importantly, however, the report shows that, inhibition of NET production, as modeled by the PAD4-null mice, prevents collateral host tissue damage, suggesting that therapeutic PAD4 inhibitors are not likely to cause host tissue damage during infection (Kolaczkowska et al. 2015).

4.5 PAD6

PAD6 expression is primarily limited to mammalian oocytes and early embryos and was first cloned and characterized because of its high expression levels in mouse eggs (Wright et al. 2003). In order to study the function of PAD6, we investigated the effects of PAD6 deletion (using a somatic knockout) on fertility and

development. We found that, while PAD6-null males were fertile, PAD6-null females were sterile (Esposito et al. 2007). Further analysis found that oocytes from these females could be fertilized at normal rates; however, the resulting embryos underwent developmental arrest at the two-cell stage (Esposito et al. 2007). This result indicated that PAD6 functions as a maternal effect gene. We then went on to investigate the potential mechanisms that caused the developmental arrest and found that the PAD-null embryos appeared to have defects in their ribosomal machinery leading to defective protein synthesis and a failure to undergo embryonic genome activation (Yurttas et al. 2008). Additionally, EM analysis of the null oocytes and early embryos found that PAD6 is required for the formation of an abundant cytoskeletal structure known as the cytoplasmic lattices (Esposito et al. 2007). We also found that microtubule dynamics were defective in the PAD6-null oocytes and that these oocytes could not properly reposition mitochondria and endoplasmic reticulum during oocyte maturation (Kan et al. 2011). Taken together, results from our PAD6 knockout studies suggested that PAD6 is a component of a large supramolecular complex (i.e., the lattices) and that this microtubule-based complex plays a critical role in protein synthesis and organelle positioning in the oocyte and early embryo. The precise mechanism by which PAD6 regulates cytoplasmic lattice assembly and function remains unclear. As opposed to the other PADs, PAD6 does not appear to be catalytically active (Taki et al. 2011). Therefore, it seems likely the role of PAD6 in these processes is structural. Given the strong association between PADs and histone citrullination, we have also tested whether histone citrullination may play a role in early development. Results showed that the pan-PAD inhibitor, Cl-amidine, suppresses histone H3 and H4 tail citrullination and, similar to that seen in PAD6-null oocytes, potently suppresses early cleavage divisions (Kan et al. 2012). We then investigated histone citrullination levels in PAD6- and PAD4-null oocytes and found that deletion of these two family members did not affect histone citrullination, suggesting that another PAD was likely catalyzing this activity in eggs and early embryos. We recently found that PAD1-specific inhibitors and morpholinos both suppressed histone citrullination and early-stage cleavage divisions, suggesting that PAD1 may play a critical role in early development via its role in histone citrullination (Zhang et al. 2016).

While we have shown that PAD6 is expressed human oocytes, until recently, it has remained unclear as to whether PAD6 plays an important role in fertility in women. However, Xu et al. recently found that PAD6 mutations appear to be the cause of infertility in several women who have failed to become pregnant following multiple IVF and ICSI cycles (Xu et al. 2016). These mutations occurred within a consanguineous family affected by a homozygous premature nonsense mutation and also in two females with compound heterozygous mutations. Consistent with what we had found in our PAD6 knockout mice, their study also found that PAD6 protein was absent from affected oocytes and that, following fertilization, all of the affected embryos arrested at the 2–4 cell stage due to embryonic genome activation failure (Xu et al. 2016). This finding was recently supported by another case reporting that a PAD6 mutation was the cause of infertility in a women that had unsuccessfully undergone multiple rounds of ICSI only to have the resulting embryos

arrest at the two-cell stage (Maddirevula et al. 2017). Taken together, these findings suggest that PAD6 mutations may be a significant cause of infertility in women.

4.6 Conclusions

The critical role that PAD enzymes play in mammalian development, physiology, and pathology is now coming to light. Our new understanding of PAD biology has been significantly advanced in recent years, in part, through the use of genetically engineered mice. Outcomes from PAD4 knockout mice indicate that PAD4 is required for citrullination in immune cells and that this activity plays an important physiological role in innate immunity. These studies also demonstrate that dysregulated PAD4 activity can lead to various pathologies. For example, the knockout studies have supported previous clinical findings that PAD4 activity plays a critical role in chronic inflammatory diseases such as rheumatoid arthritis. Additionally, these studies also highlight a novel and important role for PAD4 in thrombosis formation via its role in NET production within the thrombus scaffold. Given the prevalence of autoimmune diseases and thrombotic disorders in society, it is understandable that there is currently considerable interest in developing PAD4-specific compounds and then testing the efficacy of these compounds in a clinical setting.

Outcomes from PAD2-overexpressing and knockout mice have also proved to be informative. Studies investigating the role of PAD2 in CNS function found that, while PAD2 activity appears to be the main driver of citrullination in this tissue in both healthy and in autoimmune encephalomyelitis, it does not appear to play a critical role in CNS development or in the development of experimental autoimmune encephalomyelitis. These findings indicate that the precise functional role of PAD2 within the CNS remains to be determined. With respect to immune cells, the observation that citrullination activity was only moderately reduced in immune cells from PAD2 knockout mice suggests that the overall contribution of PAD2 to immune cell function may be less than that of PAD4. Regarding the role of PAD2 in the skin, our finding that PAD2 overexpression in the skin promotes inflammation and tumor growth demonstrates, for the first time, that PADs can function as oncogenes. Given the fact that PAD activity is strongly associated with inflammatory events, our findings also raise the possibility that PAD-mediated inflammation may promote the growth of a range of cancers.

A surprising outcome from the totality of PAD transgenic/knockout studies is the lack of a strong grossly observable phenotype in these lines. All of the PAD2, PAD3, and PAD4 mutant strains generated to date appear normal at the whole body level and are fertile. PAD6 mutant mice are also grossly normal; however, null females are infertile, due to an arrest at the two-cell stage of development. Given that, aside from PAD6, PADs are expressed in a broad range of tissues including the CNS, muscle tissue, exocrine glands, and immune cells, one would predict that the PAD mutant mice would likely acquire developmental defects that would render the offspring

nonviable. A likely explanation for this lack of effect is that one or several of the PAD isozymes compensate for the loss of another family member. In fact, several of the studies mentioned in this review noted that deletion or overexpression of one family member resulted in the up- or downregulation of another family member. Therefore, future studies aimed at deciphering how PAD isozymes compensate for each other and also how these family members potentially regulate each other's expression will be critical for understanding how PADs function at the organism level. Additionally the use of new CRISPR technologies to generate mouse lines that lack more than one PAD family member will also likely greatly contribute to our understanding of PAD physiology and pathology.

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